Accepted number	662-05- E-3740
Study number	93740

COMPANY SANITIZED DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

FINAL REPORT

A 48-hour Acute Immobilization Study of with *Daphnia magna*

March 22, 2006

STATEMENT

Sponsor

Title A 48-hour Acute Immobilization Study with *Daphnia magna*

Study number 93740

I, the undersigned, hereby declare that this report provides a correct English translation of the Final Report (Study No. 93740, issued on March 22, 2006).

Date September 20, 2006

Study Director

GLP STATEMENT

Sponsor

Title A 48-hour Acute Immobilization Study of *Daphnia magna*

Study number 93740

This study was performed in compliance with:

(1) "Standard Concerning Testing Facility Relating to New Chemical Substances" (November 21, 2003; No. 1121003, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 17, 2003, No. 3, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121004, Environmental Policy Bureau, Ministry of the Environment)

(2) "OECD Principles of Good Laboratory Practice" (November 26, 1997)

This final report reflects the raw data accurately and it has been confirmed that the test data are valid.

Date March 22, 2006

Study Director

QUALITY ASSURANCE STATEMENT

Sponsor

Title A 48-hour Acute Immobilization Study of with *Daphnia magna*

Study number 93740

It has been assured that the final report accurately describes the test methods and procedures, and that the reported results accurately reflect the raw data of the study.

The inspections of this study were carried out and the results were reported to the Study Director and the Test Facility Management by Quality Assurance as follows.

Item of inspection	Date of inspection	Date of report to Study Director and Test Facility Management
Study plan draft	February 9, 2006	February 9, 2006
Study plan	February 10, 2006	February 10, 2006
Amendment of study plan	March 9, 2006	March 9, 2006
Start of the exposure and after the exposure	February 13, 2006	February 15, 2006
	February 15, 2006	February 15, 2006
Raw data and final report draft	March 10, 2006	March 10, 2006
Final report	March 22, 2006	March 22, 2006

Date March 22, 2006

Quality Assurance Unit, Head Signed in original

CONTENTS

SUMMARY

A 48-hour Acute Immobilization Study *Daphnia magna*

<Test condition>

- Test substance

- Test organism
  Daphnia magna

- Exposure duration
  48 hours

- Test concentration
  100 mg/L and a control

- Number of organisms
  20 daphnids/test level (5 daphnids/vessel)

- Dilution water
  Dechlorinated tap water

- Type of test
  Static

- Preparation of test solution
  Test solution was prepared by stirring after test item supplied by the sponsor was mixed with dilution water directly.

- Replicates
  4 replicates/test level

- Volume of test solution
  400 mL/test level (100 mL/vessel)

- Water temperature
  20±1℃

- Light condition
  Room lamp, 16-hour light/8-hour dark

- Feeding
  No feeding

- Aeration
  No aeration

・Analysis of concentration of test item in test solution
HPLC analysis (at the start and the end of the exposure)

<Results>

・Concentration of test item in test solution (Percentage of nominal concentration)
At the start of the exposure 99.0%
At the end of the exposure 99.3%

・48-hour EC50 (Median Effective Concentration)
>100 mg/L
(The above-mentioned concentration is based on nominal concentration.)

1. Title

A 48-hour Acute Immobilization Test c with *Daphnia magna*

2. Sponsor

Name

Address

3. Testing facility

Name

Address

4. Objective

The objective of this study is to determine the acute toxicity of the test item to *Daphnia*.

5. Test method

This study was performed according to the following test methods.

(1) *Daphnia* sp., Acute Immobilization Test stipulated in the "Testing Methods for New Chemical Substances" (November 21, 2003; No. 1121002, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 13, 2003, No. 2, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121002, Environmental Policy Bureau, Ministry of the Environment)

(2) OECD Guidelines for Testing of Chemicals, Section 2 : Effects on Biotic Systems, 202 "*Daphnia* sp., Acute Immobilisation Test (Guideline 202, April 13, 2004)".

6. Applied GLP

This study was performed in compliance with:

(1) "Standard Concerning Testing Facility Relating to New Chemical Substances" (November 21, 2003; No. 1121003, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 17, 2003, No. 3, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121004, Environmental Policy Bureau, Ministry of the Environment)

(2) "OECD Principles of Good Laboratory Practice" (November 26, 1997).

7. Dates

1)	Study initiation date	February 10, 2006
2)	Experimental starting date	February 13, 2006
3)	Experimental completion date	February 15, 2006
4)	Study completion date	March 22, 2006

8. Storage of test item, raw data, etc

1) Test item

The test item* supplied by the sponsor is sealed in a storage vessel and stored in a storage room in for ten years after the receipt of notice specified under Clause 1 or Clause 2 in Article 4, Clause 2 or Clause 3 or Clause 8 in Article 4-2, and Clause 2 in Article 5-4 or Clause 2 in Article 24 or Clause 2 in Article 25-3 of "Law Concerning Examination and Regulation of Manufacture, etc. of Chemical Substances". Treatment of the item supplied by the sponsor after the storage period is discussed with sponsor. If it is not stable for the storage period, it is stored as long while it is kept stable and it is disposed with approval of sponsor.

2) Raw data and materials

Raw data, the study protocol, documents concerning the study presented by the sponsor, the final report and necessary materials are stored in archives in ten years after the receipt of the notice specified under Clause 1 or Clause 2 in Article 4, Clause 2 or Clause 3 or Clause 8 in Article 4-2, and Clause 2 in Article 5-4 or Clause 2 in Article 24 or Clause 2 in Article 25-3 of "Law Concerning Examination and Regulation of Manufacture, etc. of Chemical Substances". Treatment of raw data and materials, etc. after the storage period is discussed with the sponsor.

9. Personnel

Study Director :

Fourth testing section

Study Personal

Biology :

Analytical Chemistry :

10. Approval of final report

Study Director Date March 22, 2006

Signature Signed in original

11. Test item

has the following name etc. in this final report.

11.1 Test item

The following are within the test item information provided by the sponsor.

1) Name

2) Name (abbreviation)

3) Rational formula etc.

(1) Rational formula

(2) Molecular formula

(3) Molecular weight

11.2 Test item supplied by the sponsor

The following are within the test item supplied by the sponsor information provided by the sponsor.

1) Lot Number

RS4-56

2) Purity

99.5%(w/w)

3) Name and content of impurity

Water 0.5%(w/w)

4) Physicochemical properties etc.

(1) Appearance at normal temperature

White solid

(2) Melting point

(3) Soluble property

Oil and water soluble

(4) Stability

Stable under room temperature

Stable in water, DMSO and acetone

5) Supplier

11.3 Confirmation of test item supplied by the sponsor

It was confirmed that infrared (IR) spectrum of the test item provided by the sponsor coincided with IR spectrum analyzed in Kurume Laboratory.

11.4 Storage condition and confirmation of stability under the storage condition

(1) Storage condition

The test item supplied by the sponsor was kept in a dark place at room temperature during the test period.

(2) Confirmation of stability

The stability of the test item during the test period was confirmed by no alteration in the IR spectra of the test item before the experimental start and after the experimental completion.

12. Test materials and methods

1) Test organism

(1) Species

Daphnia magna (Clone A)

(2) Reason for selection of species

Species recommended in the test guidelines.

(3) Source

Young *daphnia* produced by parents which were cultured in the was used. *Daphnia* originally came from the University of Sheffield (Address: Sheffield S10 2UQ, United Kingdom). The parents to obtain young *daphnia* were bred in the same quality of water (dechlorinated tap water), water temperature (20±1℃), and photoperiod (16-hour light/8-hour dark) as used in the study. Parents used for the study were same lot, and their and survival rate were 17-day old and 100%. *Chlorella vulgaris* of 0.1 to 0.2 mgC/day per *daphnia* was fed to the parents once a day. A 48-hour acute immobilization test of potassium dichromate (Reagent grade, Wako Pure Chemical Industries) with the test organisms was conducted (December 2-4, 2005) to confirm the reproducibility of the test conditions. The 48-hour EC50 of the potassium dichromate was 0.283 mg/L. This value was within the normal range of the potassium dichromate in (mean ± 2S.D.: 0.116 to 0.351 mg/L) [mean ± S.D.: 0.233± 0.059 mg/L (n=52)].

(4) Selection of young daphnids

Less than 24-hour old daphnids were used for the test.

(5) Allocation to the test groups

Test organisms were placed at random to each test vessel.

2) Dilution water

Dechlorinated tap water, aerated sufficiently and controlled temperature, was used. Some chemical characteristics of the dilution water are listed in Appendix 1.

3) Test apparatus and equipment

(1) Test apparatus

Test vessel : 100 mL glass beaker

The test vessels were covered with lid in order to prevent dust, and volatilization of the test solution.

(2) Test equipment

Water bath : Plastic tank

Warming/cooling unit (Type HCA250, Sato craft)

4) Test conditions

(1) Conditions of exposure

① Type of test

The test organisms were exposed to the test solution containing the test item. The test solutions were not renewed, as static regime.

②Exposure duration

48 hours

③Test concentration

One exposure level (the maximum concentration on applied test guideline : 100 mg/L) was used in the test from the results of preliminary tests. The test concentration was expressed as value corrected by the purity (99.5%) of the test item. The results of preliminary tests are shown in Additional data.

④Control

Only the dilution water was used for a control.

⑤Replicates

4 replicates/test level

⑥Number of organisms

20 daphnids/test level (5 daphnids/vessel)

⑦Volume of test solution

400 mL/test level (100 mL/vessels)

(2) Conditions of test environment

①Water temperature

20±1℃

②Dissolved oxygen concentration

This study was performed in the condition where dissolved oxygen concentration was at least 60% or more of the saturate concentration at the water temperature used. No aeration was used for the test during the exposure.

③pH

This study was performed without adjusting pH.

④Light

16-hour light/8-hour dark photoperiod daily with room lamp

⑤Feeding

Test organisms were not fed during the exposure.

5) Preparation of test solution

Correction with the purity(99.5%) was applied to the preparation of the test solution.

Desired amount of test item supplied by the sponsor was stirring after addition of dilution water in the container for preparation. The prepared test solution was divided into each test vessel.

Amount of the test item supplied by the sponsor in each exposure level is shown below.

Test level (mg/L)	Amount of test item supplied by the sponsor (mg/995 mL)
Control	–
100	100

6) Observation and measurements

(1) Observation of test organisms

Immobility and symptom were observed at 24 and 48 hours after the exposure. Daphnids were considered immobile if they were not able to swim within 15 seconds after gentle agitation of the test vessel.

(2) Appearance of the test solution

Appearance of the test solutions was observed at the start and the end of the exposure.

(3) Water quality

Dissolved oxygen concentration, pH and water temperature of the test solution were measured at the start and the end of the exposure. At the start of the exposure, another solution sampled from the container for preparation was used for the measurement. At the end of the exposure, the measurement was carried out for one of four test vessel in each level. The dissolved oxygen concentration measurements were carried out on an oxygen meter (Model 58, Yellow Springs Instruments). The pH measurements were carried out on a pH meter (Model HM-14P, DKK-TOA). The water temperature measurements were carried out on a calibrated red alcohol thermometer of glass stick type.

(4) Concentration of test item in test solution

The concentration of the test item in the test solution was measured at the start and the end of the exposure. At the start of the exposure, another solution sampled from the container for preparation was used for analysis. At the end of the exposure, the equal volume of the test solution was taken out from the middle layer of the test solution in test vessels in each level and was mixed and used for analysis. The concentration of the test item was analyzed by high-performance liquid chromatography (HPLC). Method of analysis and result of measurement of test item concentration are shown in Appendix 2, and analytical calibration curve and chromatograms are shown in Appendix 3.

7) Calculating method of EC50*

The EC50 value was estimated as "> test concentration" since no less than 50% immobility was not observed in the present exposure level.

The results of the study were estimated based on nominal concentrations because the measured concentrations of the test item in the test solution during the exposure were kept within the range of ±20% of the nominal concentrations.

* EC50 (Median Effective Concentration) is the concentration which causes 50% immobility of tested population during the exposure.

8) Study validity

(1) In the control, not more than 10% of the daphnids should have been immobilized or floating in the surface of the water during the exposure period.

(2) Dissolved oxygen concentration must be maintained as at least 60% or more of the saturate concentration at the water temperature in the test during the exposure.

9) Treatment of numerical values

Values were rounded off in accordance with JIS Z 8401 rule B.
(JIS ; Japanese Industrial Standards)

13. Results and consideration

1) Immobility

No abnormal responses were observed in the present test concentration during the exposure. Immobility at 24 and 48 hours are shown in Table 1. Immobility in the control during the exposure was 0%, which meets the criterion for the validity of the test (i.e. less than 10%), and no floating daphnids were observed.

2) Observed abnormal response

There was no abnormal response in the control. The following results of observation were based on the comparison with the control organisms. No abnormal responses were obtained in the present exposure level during the exposure. Observed abnormal response during the exposure is shown in Table 2.

3) Observation and measurement of test solution

(1) Appearance of test solution

The test solutions were clear and colorless at the start of the exposure. The appearance kept until the end of the exposure.

(2) Water quality of test solution

The measured values of dissolved oxygen concentration, pH and water temperature during the exposure ranged from 8.7 to 8.8 mg/L, 7.7 to 7.8 and 20.2 to 20.7℃, respectively. Water qualities of the test solutions are shown in Table 3. The measured values of dissolved oxygen concentration met the criterion for the validity of the test (at least 60% or more of saturate concentration* at the water temperature).

* Saturated dissolved oxygen concentration (19 to 21℃): 9.01 to 8.68 mg/L(JIS K 0102)

(3) Concentration of test item in test solution

The measured concentrations of the test item in the test solution were 99.0% of the nominal concentration at start of the exposure, and they were 99.3% at the end of exposure. These measured concentrations of the test item were not kept within ±20% of the nominal concentration. The results of the measured concentrations of the test item are shown in Appendix 2.

4) EC50

Both the 24-hour and 48-hour EC50 of *Daphnia magna* were >100 mg/L. The EC50s at every observation time are shown in Table 4.

5) Consideration

The present study was conducted as a limit test to confirm that the test item has no effects on the test organisms at the maximum concentration on applied test guideline (100 mg/L). The measured concentrations of the test solution were kept within the range of ±20% of the nominal concentration, and the environmental conditions were also within the suitable range.

Therefore, it is concluded that the results in this study were appropriately obtained in the suitable conditions where the present test was performed according to the test guidelines.

14. Factors that affected the reliability of the test results

No adverse effect on the reliability of this study was observed.

Table 1 Immobility

Nominal concentration (mg/L)		Immobility(%)			
		24 hours		48 hours	
		Replicate	Test level	Replicate	Test level
Control	A	0	0	0	0
	B	0		0	
	C	0		0	
	D	0		0	
100	A	0	0	0	0
	B	0		0	
	C	0		0	
	D	0		0	

Table 2 Observed abnormal response

Nominal concentration (mg/L)	Observed abnormal response	
	24 hours	48 hours
Control	—	—
100	—	—

—: Normal (No abnormal response)

Table 3 Water quality parameters measured in test solution

Nominal concentration (mg/L)	Dissolved oxygen concentration (mg/L)		pH		Temperature (℃)	
	At the start	At the end	At the start	At the end	At the start	At the end
Control	8.7	8.8	7.7	7.7	20.2	20.7
100	8.7	8.8	7.7	7.8	20.2	20.7

Table 4 EC50 to *Daphnia magna*

Exposure duration	EC50 (mg/L)	95% confidence limits(mg/L) (Slope of the dose-response curve)	Statistical procedure used for determination of EC50
24 hours	>100	— (—)	—
48 hours	>100	— (—)	—

—: Not obtained

Appendix 1

Water quality of dilution water

Water quality of dilution water (Sampling on January 11, 2006)

Parameter	Unit	Results	Lower limit of determination
Hardness (as $CaCO_3$)	mg/L	41.7	0.1
Suspended substance	mg/L	< 1	1
pH	—	7.9(19℃)	—
Organic carbon	mg/L	0.8	0.1
Chemical oxygen demand	mg/L	1.0	0.5
Free chlorine	mg/L	< 0.02	0.02
Ammonium nitrogen	mg/L	0.01	0.01
Cyanide	mg/L	< 0.01	0.01
Alkalinity	mg/L	36	1
Electric conductivity	mS/m	17.1	—
Organic phosphorous	mg/L	< 0.1	0.1
Alkyl mercury	mg/L	< 0.0005	0.0005
Total mercury	mg/L	< 0.0005	0.0005
Cadmium	mg/L	< 0.001	0.001
Hexavalent chromium	mg/L	< 0.02	0.02
Lead	mg/L	< 0.005	0.005
Arsenicum	mg/L	< 0.001	0.001
Boron	mg/L	0.07	0.02
Fluorine	mg/L	0.1	0.1
Iron	mg/L	< 0.01	0.01
Copper	mg/L	< 0.005	0.005
Cobalt	mg/L	< 0.001	0.001
Manganese	mg/L	< 0.01	0.01
Zinc	mg/L	< 0.01	0.01
Aluminum	mg/L	0.063	0.001
Nickel	mg/L	< 0.001	0.001
Silver	mg/L	< 0.0001	0.0001
Sulfate ion	mg/L	16.2	0.1
Chloride ion	mg/L	14	1
Sodium	mg/L	13.4	0.01
Potassium	mg/L	3.5	0.01
Calcium	mg/L	11.2	0.01
Magnesium	mg/L	3.3	0.01
1,2-dichloropropane	mg/L	< 0.0001	0.0001
Chlorothalonil	mg/L	< 0.0001	0.0001
Propyzamide	mg/L	< 0.0001	0.0001
Chlornitrofen	mg/L	< 0.0001	0.0001
Simazine	mg/L	< 0.001	0.001
Thiobencarb	mg/L	< 0.0001	0.0001
Diazinon	mg/L	< 0.0001	0.0001
Isoxathion	mg/L	< 0.0001	0.0001
Fenitrothion	mg/L	< 0.0001	0.0001
EPN	mg/L	< 0.0001	0.0001
Dichlorvos	mg/L	< 0.0001	0.0001
Iprobenfos	mg/L	< 0.0001	0.0001
PCB	mg/L	< 0.0005	0.0005

Appendix 2

Method of analysis and result of measurement of test item concentration

1. Method of analysis of test item concentration

1) Pretreatment of test solution

The test solutions sampled were used as the samples for analysis after no treatment.

2) Method of analysis

The pretreated samples for analysis were quantitatively analyzed by high-performance liquid chromatography (HPLC) under the following conditions to determine the concentration of the test item. The concentration of the test item in the samples for analysis was proportionally calculated by comparing each peak area of the test item on the chromatogram with that of a standard solution of known concentration. The obtained some chromatograms are shown in Appendix 3.

Analytical conditions

Instrument	High-performance liquid chromatograph SHIMADZU LC-2010A_{HT}
Column	L-column ODS (Chemicals Evaluation and Research Institute) 15 cm×2.1 mm I.D.
Column temp.	40℃
Eluent	Acetonitrile/10 mmol/L tetra-*n*-butylammonium phosphate solution* 55/45(v/v)
Flow rate	0.2 mL/min
Wave length	
Injection volume	5 μL
Sensitivity	
Detector	0.5 AU/V

* It was prepared from tap water treated with a ultra pure water system.

3) Preparation of standard solution

The standard solution to determine the concentration of the test item in the sample for analysis was prepared as follows. The standard solution was prepared with correcting by the purity (99.5%) of test item.

Test item supplied by the sponsor of 100.5 mg was precisely weighed with an electronic balance and dissolved in dechlorinated tap water to prepare 1,000 mg/L of test item solution. The standard solution of 100 mg/L was then prepared from this solution by dilution with dechlorinated tap water.

4) Calibration curve

The standard solutions of 10.0, 50.0, 100 and 200 mg/L were prepared for analysis by the same procedure as described in 3). These solutions were analyzed according to the quantitative analytical conditions described in 2). A calibration curve was drawn from the relationship between the concentrations of test item and the peak area on the chromatogram respectively, the quantitative correlation was confirmed. The calibration curve and regression equation are shown in Appendix 3. The determination-limit of the test item in the test solution was the lowest determination-limit (10.0 mg/L) of the standard solutions within the range of the calibration confirmed.

2. Results of the measurement

The results of the measured concentrations of the test item in the test solution are shown below.

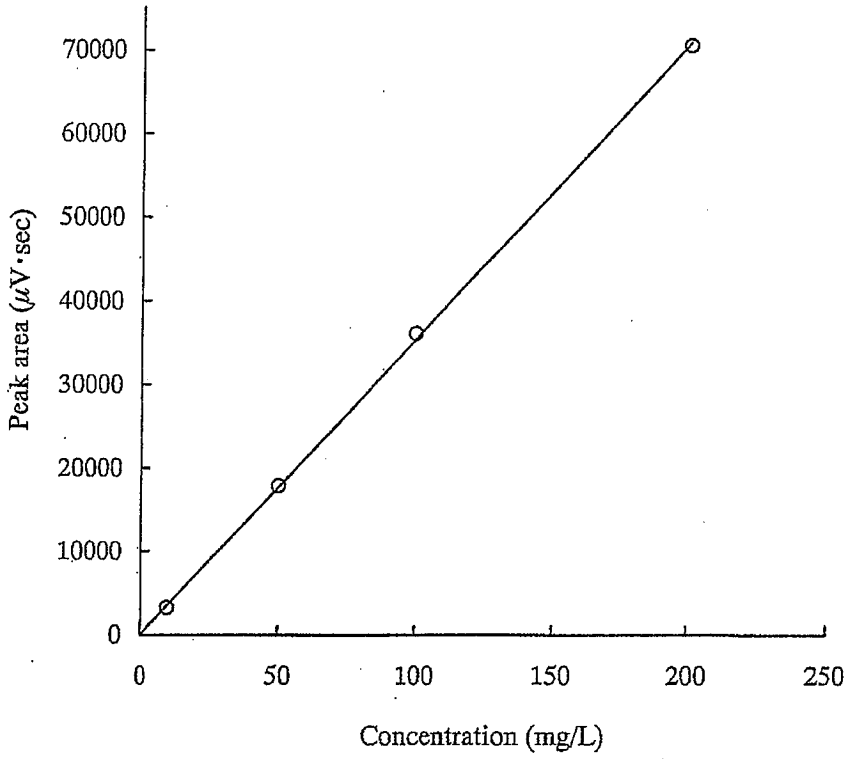
Appendix table 2-1. Measured concentrations of test item in test solutions

Nominal concentration (mg/L)	Measured concentration of test item (mg/L) (Parcentage of nominal concentration)		
	At the start	At the end	Mean*
Control	n.d.	n.d.	—
100	99.0 (99.0)	99.3 (99.3)	99.2 (99.2)

n.d. : Not determined (<10.0 mg/L)
* The value is expressed as geometric mean.

Appendix 3

Calibration curve and chromatograms



$y = 354x$
$r = 1.00$

Appendix figure 3-1. Calibration curve of HPLC.

Additional data

Results of preliminary tests

1. Solubility of test item in dilution water
   1) Examination
      (1) Method

Solubility of the test item was confirmed by mixing them the test item supplied by the sponsor and dilution water to produce concentration of 100 mg/L

      (2) Result

The test solution was clear and colorless, and no insoluble material was observed. Therefore, it was concluded that the test item in dilution water at 100 mg/L was dissolved.

   2) Summary

It was decided that solubility of the test item to dilution water was not measured in this study because the solubility in dilution water was greater than 100 mg/L.

2. Effect on test organism
   1) Preliminary test-1
      (1) Method

The test solutions were prepared by mixing them the test item supplied by the sponsor and dilution water. The effect on the test organism was confirmed by exposing the organisms to the test solution.

      (2) Result

Test level (mg/L)	24 hours		48 hours	
	Immobility (%)	Other abnormal response	Immobility (%)	Other abnormal response
Control	0	—	0	TSWs RA
1.00	0	—	0	TSWs RA
10.0	0	—	0	TSWs RA
100	0	—	0	RA

Type of test: static
Test organisms: Five daphnids/test level
Preparation of test solution: The test solutions were prepared with the stock solution which was prepared by mixing the test item supplied by the sponsor with dilution water and stirring. The stock solution was not expressed as value corrected by the purity (99.5%) of the test item.
—: Normal (No abnormal response)
Abbreviation of symptoms
RA : Reduced activity
TSWs : Trapped at the surface of water but capable of swimming

It was considered that the abnormal responses at the exposure levels were not effect of the test item to the test organism. Therefore, it was expected that the test item has no effect on the test organism at the maximum concentration on applied the test guideline.

2) Preliminary test-2

(1) Method

The effect on the test organism was reconfirmed by exposing the organisms at 100 mg/L. And the concentration of the test item in the test solution during the exposure was measured with time.

(2) Result

Nominal concentration (mg/L)	24 hours		48 hours	
	Immobility (%)	Other abnormal response	Immobility (%)	Other abnormal response
100	0	—	0	—

Type of test: static

Test organisms: Ten daphnids/test level

Preparation of test solution: The test solutions were prepared with the stock solution which was prepared by mixing the test item supplied by the sponsor with dilution water and the stirring. The stock solution was not expressed as value corrected by the purity (99.5%) of the test item.

—: Normal (No abnormal response)

Nominal concentration (mg/L)	Measured concentration of test item (mg/L) (Percentage of nominal concentration)		
	At the start (0-hour)	After 24-hour	At the end (After 48-hour)
100	106 (106)	105 (105)	102 (102)

The abnormal response was not observed to test organisms. The measured concentrations of the test item in the test solution during the exposure were kept about 100% of the prepared concentrations.

3) Summary of the effect on test organism (results of preliminary tests)

The abnormal response was not observed to test organisms at the maximum concentration on applied test guideline (100 mg/L) and the measured concentration of the test item in the test solution were stabilized. Therefore one exposure level (the maximum concentration on applied test guideline: 100 mg/L) was used in the study as static regime.